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Microsatellite (SSR) markers assisted characterization of rice (*Oryza sativa* L.) genotypes in relation to salt tolerance

Bibha Rani*and V K Sharma

Department of Biotechnology and Molecular Biology, Dr Rajendra Prasad Central Agricultural University, Pusa, Bihar, India.

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Under present investigation a total of 18 rice genotypes with diverse genetic background were taken for screening at seedling stage and its molecular characterization using SSR markers. Selected rice genotypes were exposed to five salinity levels (0, 2, 4, 6 and 8 dSm^{-1}) at seedling stage. Modified standard evaluation score (SES) at seedling stage identified 7 highly tolerant, 2 tolerant and 8 moderately tolerant genotypes. The susceptible genotype was IR64 with score 7. Considering 4 other morpho-physiological characters (germination percent, K/Na ratio, shoot and root length, shoot and root fresh weight, shoot and root dry weight) studied at seedling stage, the genotype CSR2K-262 showed greater tolerance among all the 18 genotypes. For molecular characterization a total of 44 SSR markers were selected. The average number of alleles per locus was 9.3 indicating greater magnitude of diversity among plant materials. The average PIC value 0.767 confirmed that the markers used were highly informative. The cluster analysis grouped the 18 genotypes into three major clusters. Cluster I was the largest with 9 genotypes with all highly tolerant and tolerant genotypes, Cluster II comprised 8 genotypes with all tolerant and moderately tolerant and Cluster III comprised of 1 genotype (IR64) identified as susceptible one.

Keywords: Rice, salinity, SES, SSR, salt tolerance

Introduction

Salinity is one of the major constrains to productivity in rice growing areas worldwide. The productivity of rice is declining and unable to meet out for growing population due to adverse abiotic and soil factors, in addition to biotic factors. The possible ways to mitigate the adverse effects of salinity on rice production are reclamation of soil and breeding new varieties suitable for saline soils. However, reclamation is not always practically feasible as it needs more financial investment and laborious man power support. The other possible strategy is breeding to enhance salinity tolerance, but it has been slow due to limited knowledge about the genetics of salt tolerance, inadequate screening techniques and low selection efficiency.

Among abiotic stresses, salinity is foremost and second most widespread problem causing reduction in growth and productivity of crop plants[1-2]. Salinity is an environmental condition which adversely affects the physiological processes of crop plants and severely affects crop production. The adverse effects may be attributed to non availability of water and disturbance in nutrient uptake causing deficiency and ion-toxicity to plants. Plants growing under saline condition invariably face increased concentrations of toxic ions in their tissues resulting from increased uptake of ions, mainly Na and Cl under salinity.

Generally, salinity tolerance is considered to be a polygenic trait. Screening rice germplasm to locate salt tolerance genes for use in improving the currently grown varieties is of continuous importance to plant biotechnologists[3]. Conventional breeding is time consuming and depends on environmental conditions. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in rice breeding. Integration of breeding with recent marker assisted selection technology makes it feasible to analyze quantitative traits at early seedling stage, which fasten the breeding programme. Molecular markers can be used to tag quantitative trait loci (QTL) and to evaluate their contribution to the phenotype by selecting favorable alleles at these loci in order to accelerate genetic improvement. Recent progress and technical advances in molecular marker technology permit reduction of time and accuracy of breeding where pronounced effects of environment lead to poor selection efficiency.

*Author for correspondence:
Mobile: 8986931371
bibha9rani@gmail.com

Molecular markers are target sites in the genome that differ between individuals of a population. These differences can occur in deoxyribonucleic acid that codes for specific genes, or usually in the vast areas of intergenic region[4]. Molecular markers provide information that can help to define the distinctiveness of germplasm and their ranking according to the number of close relatives and their phylogenetic position. Among the molecular markers, microsatellite markers or simple sequence repeats (SSRs) based markers are known to be highly polymorphic, more reproducible, co-dominant, abundant and distributed throughout the genome. These markers reveal polymorphism due to variation in the length of SSRs that can be easily and economically assessed by polymerase chain reaction using primers specific to the unique flanking sequences of the SSR and polymorphic amplified fragments can be produced due to difference in the number of the repeat units. One of the well established features of SSR loci is their hyper-variability, which is associated with the expansion potential of the SSR motif itself. Because of highly polymorphic nature, SSR markers offer an easy, accurate, and quantifiable measure of the genetic variation[5]. This feature, in combination with the co-dominant profiles and the potential for automation, has contributed to the widespread use of these markers in a wide range of genetic studies.

Carefully chosen set of SSR markers is playing an important role to identify gene (s) for salt tolerance that can be helpful for plant breeders to develop new cultivars, besides facilitating an unbiased assessment of genetic differentiation, an unambiguous description of rice cultivars and development of unique molecular profiles of rice genotypes. The present study focused on the analysis of morpho-physiological attributes and SSR markers based molecular profiles in relation to salt tolerance status of rice genotypes. Keeping this into consideration in the light of aforesaid information, the present study was undertaken with the following objectives:

a) To screen the selected rice genotypes for the different level of salinity at seedling stage based on SES and other morpho-physiological parameters;
b) To characterize the rice genotypes with different extent of adaptation to salt stress using microsatellite marker's and
c) To examine the nature of microsatellite markers based polymorphism for identification of informative markers in order to discriminate rice genotypes.

Materials and Methods

Plant Material

Eighteen rice genotypes with diverse genetic background including four released cultivars, one landrace and thirteen advanced breeding lines were selected (Table 1). Most of the genotypes under investigation were newly developed inbred lines under the STRASA (Salt Tolerant Rice for Africa and South Asia) project for evaluation of their extent of adaptability to salt stress and release of most promosing variety. Among released cultivars CSR 36 taken as salt tolerant check and IR 64 taken as salt susceptible check.

Screening at Seedling Stage for Salt Stress Tolerance

Petriplate experiment for the screening at 2-3 leaf stage was executed under the controlled environment. The experiment was done in completely randomized design (CRD) with two replications for recording the effects of salt stress on rice genotypes at seedling stage. Salinized and non-salinised set ups were maintained with two replications. The entries were exposed to five salinity levels (0, 2, 4, 6 and 8 dSm^{-1}). Seeds of the 18 rice genotypes were soaked in five levels of salt solutions with two replications for 36 hrs and the allowed to germinate on petriplate with

Table 1— List of 18 rice genotypes used for diversity analysis

S. No.	Name of genotypes	Source	Status
1.	CSR36	CSSRI, Karnal	Released variety
2.	CSR27-192	CSSRI, Karnal	Inbred line
3.	CST7-1	West Bangal	Released variety
4.	CSR2K-242	CSSRI, Karnal	Inbred line
5.	PNL4-35-20-4-1-4	P.A.U, Panjab	Inbred line
6.	NDRK11-1	NDUAT, Faizabad	Inbred line
7.	NDRK11-3	NDUAT, Faizabad	Inbred line
8.	CR2814-2-4-3-1-1-1	CRRI, Cuttack	Inbred line
9.	CSR2K-262	CSSRI, Karnal	Inbred line
10.	CR2218-64-1-327-4-1	CRRI, Cuttack	Inbred line
11.	NDRK 11-7	NDUAT, Faizabad	Inbred line
12.	CSR2K-219	CSSRI, Karnal	Inbred line
13.	KALANAMAK	Eastern UP	Local Landrace
14.	NDRK 11-5	NDUAT, Faizabad	Inbred line
15.	NDRK 11-6	NDUAT, Faizabad	Inbred line
16.	NDRK 11-4	NDUAT, Faizabad	Inbred line
17.	RAU 1-1648	RAU, Pusa	Released variety
18.	IR 64	IRRI, Phillipins	Released variety

moisten filter paper supplied with Yoshida nutrient solution. The modified standard evaluation system (SES) was used in rating the visual symptoms of salt toxicity (IRRI, 1997)[6] (Table 2). Visual scoring was done at 8 dSm^{-1} salinity level according to Table 2. Other observations recorded at seedling stage were germination percentage, shoot and root length (Newman's method)[7], shoot and root fresh weight, shoot and root dry weight and K/Na ratio according to Zasoski and Burau (1977)[8]. Percent reduction over control (%ROC) at 8 dSm^{-1} for shoot and root fresh weight and dry weight were also calculated. Germination percentage was calculated by using formula:

$$\frac{\text{No. of seeds germinated}}{\text{Total number of seeds taken}} \times 100$$

Molecular Characterization and Genotyping of Rice Genotypes

Genomic DNA was isolated from 6 months old rice grains/seeds (10 grains) using method described by Rani and Sharma (2016)[9]. A total of 44 primers were used in the study (Table 3). The primers were selected

Table 2 — Modified standard evaluation score for visual salt injury at seedling stage

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant (HT)
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled	Tolerant (T)
5	Growth severely retarded, most leaves rolled; only a few are elongating	Moderately tolerant (MT)
7	complete cessation of growth; most leaves dry; some plants dying	Susceptible (S)
9	Almost all plants dead or dying	Highly Susceptible (HS)

Table 3 — List of forty four primers utilized for amplification of rice genomic DNA extracted from eighteen entries used in the present study

Locus	Chr. No.	Primer sequence (5'-3') F → / R ←	Repeat Motif	Annealing temp. (°C)	ESP
RM2	7	ACGTGTCACCGCTTCCT ATGTCCGGGATCTCATCG	$(GA)^{13}$	55	61
RM4	11	TTGACGAGGTCAGCACTGAC AGGGTGTATCCGACTCATCG	$(GA)^{16}$	55	72
RM5	1	TGCAACTTCTAGCTGCTCGA GCATCCGATCTTGATGGG	$(GA)^{14}$	55	66
RM9	1	GGTGCCATTGTCGTCCTC ACGGCCCTCATCACCTTC	$(GA)^{15}GT(GA)^{2}$	55	72
RM11	7	TCTCCTCTTCCCCCGATC ATAGCGGGCGAGGCTTAG	$(GA)^{17}$	55	70
RM14	1	CCGAGGAGAGGAGTTCGAC GTGCCAATTTCCTCGAAAAA	$(GA)^{18}$	55	75
RM20	12	ATCTTGTCCCTGCAGGTCAT GAAACAGAGGCACATTTCATTG	$(ATT)^{14}$	55	84
RM24	1	GAAGTGTGATCACTGTAACC TACAGTGGACGGCGAAGTCG	$(GA)^{29}$	55	98
RM25	8	GGAAAGAATGATCTTTTCATGG CTACCATCAAAACCAATGTTC	$(GA)^{18}$	55	79
RM113	1	CACCATTGCCCATCAGCACAAC TCGCCCTCTGCTGCTTGATGGC	$(CA)^{8}$	65	60
RM140	1	TGCCTCTTCCCTGGCTCCCCTG GGCATGCCGAATGAAATGCATG	$(CT)^{12}$	64	68
RM204	6	GTGACTGACTTGGTCATAGGG GCTAGCCATGCTCTCGTACC	$(CT)^{44}$	55	129
RM210	8	TCACATTCGGTGGCATTG CGAGGATGGTTGTTCACTTG	$(CT)^{23}$	55	84
RM219	9	CGTCGGATGATGTAAAGCCT CATATCGGCATTCGCCTG	$(CT)^{17}$	55	72
RM220	10	GGAAGGTAACTGTTTCCAAC GAAATGCTTCCCACATGTCT	$(CT)^{17}$	55	74
RM223	11	GAGTGAGCTTGGGCTGAAAC GAAGGCAAGTCTTGGCACTG	$(CT)^{25}$	55	90

(*Contd.*)

Locus	Chr. No.	Primer sequence (5'-3') F → R ←	Repeat Motif	Annealing temp. (°C)	ESP
RM242	12	ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG	$(AAG)^{8}(AG)^{13}$	55	90
RM253	13	TCCTTCAAGAGTGCAAAACC GCATTGTCATGTCGAAGCC	$(GA)^{25}$	55	89
RM261	14	CTACTTCTCCCCTTGTGTCG TGTACCATCGCCAAATCTCC	$C^{9}(CT)^{8}$	54	65
RM281	15	ACCAAGCATCCAGTGACCAG GTTCTTCATACAGTCCACATG	$(GA)^{21}$	55	83
RM292	16	ACTGCTGTTGCGAAACGC TGCAGCAAATCAAGCTGGAA	$(GT)^{10\text{-}6\text{-}}(TGA)^{2}TGT(TGA)^{4}$	55	85
RM333	17	GTACGACTACGAGTGTCACCAA GTCTTCGCGATCACTCGC	$(TAT)^{19}(CTT)^{19}$	55	144
RM336	18	CTTACAGAGAAACGGCATCG GCTGGTTTGTTTCAGGTTCG	$(CTT)^{18}$	55	94
RM490	19	ATCTGCACACTGCAAACACC AGCAAGCAGTGCTTTCAGAG	$(CT)^{13}$	55	66
RM493	20	TAGCTCCAACAGGATCGACC GTACGTAAACGCGGAAGGTG	$(CTT)^{9}$	58	67
RM562	21	CACAACCCACAAACAGCAAG CTTCCCCCAAAGTTTTAGCC	$(AAG)^{13}$	55	79
RM1287	22	GTGAAGAAAGCATGGTAAATG CTCAGCTTGCTTGTGGTTAG	$(AG)^{17}$	56	75
RM3395	23	ACCTCATGTCCAGGTGGAAG AGATTAGTGCCATGGCAAGG	$(CT)^{17}$	56	74
RM3412	24	AAAGCAGGTTTTCCTCCTCC CCCATGTGCAATGTGTCTTC	$(CT)^{17}$	55	74
RM7025	25	TGCGAAGTAACAAGCCTGTG GCAAAGGTTGTGTGAAGGAG	$(AAAT)^{6}$	54	64
RM8094	26	AAGTTTGTACACATCGTATACA CGCGACCAGTACTACTACTA	$(AT)^{31}$	55	104
RM10665	27	CCTGCTGCAATTGATGACAAGC TGGACAGAATGAAGCATCTGTGG	$(ATAC)^{6}$	57	69
RM10694	28	TTTCCCTGGTTTCAAGCTTACG AGTACGGTACCTTGATGGTAGAAAGG	$(AC)^{18}$	56	84
RM10745	29	TGACGAATTGACACACCGAGTACG ACTTCACCGTCGGCAACATGG	$(TATG)^{9}$	65	81
RM10748	30	CATCGGTGACCACCTTCTCC CCTGTCATCTATCTCCCTCAAGC	$(AG)^{14}$	60	71
RM10764	31	AGATGTCGCCTGATCTTGCATCG GATCGACCAGGTTGCATTAACAGC	$(AT)^{28}$	65	103
RM10772	32	GCACACCATGCAAATCAATGC CAGAAACCTCATCTCCACCTTCC	$(CTT)^{16}$	56	92
RM10793	33	GACTTGCCAACTCCTTCAATTCG TCGTCGAGTAGCTTCCCTCTCTACC	$(ATAG)^{7}$	63	76
RM10825	34	GGACACAAGTCCATGATCCTATCC GTTTCCTTTCCATCCTTGTTGC	$(AAG)^{10}$	59	76
RM10864	35	GAGGTGAGTGAGACTTGACAGTGC GCTCATCATCCAACCACAGTCC	$(GT)^{27}$	65	100
RM11008	1	TTTGGATGGTCATTAGCCTCTGG ATCAACCTTGCATGCTGTCTTCC	$(TTC)^{12}$	58	82
RM25092	10	CTATCTCCCTTGATGCGTACATGC AAATCAGCGCGTGACAATTCG	$(TG)^{15}$	60	75
RM25217	10	TGGCAGCCTCTATGTTAGACC GATGCATATCGGTGATTTGG	$(ACAT)^{14}$	60	97
RM25519	10	GGTGATTAATTACTGGTCGGAAGG GCTGGTTTGATCGGAATTACAGG	$(TA)^{42}$	61	131

on the basis of their linkage to the QTL for salt tolerance present on different chromosomes after extensive review from different research papers. The forty four SSR markers cover chromosome no. 1, 4, 6, 7, 8, 9, 10, 11, 12. Each PCR reaction carried out with 15.0 µl reactions containing 2 µl 5X buffer, 0.50 µl dNTPs, 1 µl primer forward, 1 µl primer reverse, 0.15 µl *Taq* polymerase, 0.10 µl $MgCl_2$ (10 mM), 8.25 µl ddH_2O and 2.0 µl of each template DNA samples. PCR profile was maintained as initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48-60°C for 1 min and polymerization at 72°C for 2 min; and final extension by 5 min at 72°C. Then electrophoresis in 2% agarose gel was done after polymorphism in the PCR products and stained in ethidium bromide. Banding patterns were visualized with ultraviolet gel documentation system. The polymorphism information content (PIC) of the SSR primer pairs was calculated according to the formula described by Aderson *et al*, (1993)[10] as follows:

$$PIC_i = 1 - \sum_{j=1}^{k} P^2 ij$$

Where, k is the total number of alleles detected for a marker,

P_{ij} is the frequency of the j^{th}allele for i^{th} marker and summation extends over k alleles.

Analysis of SSR Markers Based Divergence

The polymorphism in respect of SSR was recorded on the basis of presence or absence of the SSR bands in different entries. The different alleles amplified were identified on the basis of their size (base pairs or bps). A 1/0 matrix for the presence and absence of all the alleles in the genotypes was produced. A pair wise genetic similarity co-efficient matrix between all possible pairs of genotypes was generated using the simple matching (SM) similarity coefficient (Sokal and Michener, 1958)[11]. SM similarity coefficient = a+d/(a+b+c+d)

Where,

a = Number of bands between J^{th} and K^{th} genotypes

b = Number of bands present in J^{th} genotype but absent in K^{th} genotype

c = Number of bands absent in j^{th} genotype but present in K^{th} genotype

d = Number of bands absent in both J^{th} and K^{th} genotypes

Cluster analysis was performed using the data on similarity coefficients. The method used for tree building in the cluster analysis involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering based on SM coefficients. The dendrogram based on similarity indices was obtained by unweighted pair-group method using arithmetic mean (UPGMA). Principle component analysis (PCA) based on SSR was carried out using eigen vectors (the DCENTRE and EIGEN module of NTSYS-pc) for differentiation of rice genotypes projected on 2-D scatterplots. Analysis was performed with the help of NTSYS-pc 2.10e software program (Rholf, 2000)[12]. The nature of diversity between the salt tolerant and salt susceptible genotypes from amongst the entries under evaluation in the present investigation was assessed by identifying the clusters at appropriate phenon levels.

Results

Screening of Rice Genotypes for Salt Tolerance at Seedling Stage

Screening of germplasm at seedling stage is readily acceptable as it is based on simple criterion of selection; it provides rapid screening which is difficult at vegetative and reproductive stage[13-15]. Screening under controlled condition has the benefit of reduced environment effects and difficulties associated with soil related stress factors[16-18]. All genotypes were grown robustly and showed uniform green colour and height in the non-salinized condition. In salinized condition, all the tested genotypes with the exception of IR64 showed considerable variation in phenotypes due to salt toxicity ranging from score 1 (highly tolerant) to score 5 (moderately tolerant). Seedlings grown in salinized condition showed different visual symptoms such as whitish leaf tips, leaf rolling, reduction in root and shoot growth. Evaluation of physiological response of various genotypes at seedling stage under saline stress identified 7 highly tolerant genotypes (CSR27-192, CR2814-2-4-3-1-1-1, CR2218-64-1-327-4-1, CSR2K-219, Kalanamak, NDRK11-6, NDRK11-4) whose performance was almost same as CSR36 (tolerant check), 2 genotypes showed tolerant response to salinity and 8 genotypes showed moderate response to salinity. The susceptible genotype was IR64 with score 7 (Table 4) (Fig. 1).

In the present investigation a continuous increase in K/Na ratio was observed with increasing salt concentration in all the genotypes except IR64 in which a decrease in K/Na ratio was found with increase in salinity which confirms its susceptibility to salt stress (Fig. 2). At 8 dSm^{-1}salinity treatment, the genotypes

(except IR64) recorded per cent increase over control showing their tolerance to salinity. The genotypes CST7-1, CSR2K-262 and RAU1-1648 showed higher increase per cent than tolerant check CSR36 showing their higher degree of tolerance to salinity with increase in concentration of salt.

Shoot length and root length were also recorded to decrease in all the genotypes with increase in salinity. Shoot length recorded higher decrease per cent than root length in almost all the genotypes at 8 dSm^{-1} salt concentration, while the highest decrease in both shoot length and root length was recorded for IR64 showing its highest susceptibility to salinity among all the genotypes under investigation. In case of shoot length, tolerance check CSR36 recorded least reduction, while the genotypes CSR2K-262, PNL4-35-20-4-1-4, CSR27-192, NDRK11-1, NDRK11-3, CR2814-2-4-3-1-1-1 and CSR2K-262 showed higher level of tolerance than tolerant check in terms of root length at seedling stage (Fig. 3 & 4).

Table 4 —Visual scoring of rice genotypes under salinized condition (EC 8 dSm^{-1}) at seedling stage

Sl. No.	Genotypes	SES scoring	Tolerance
1.	CSR36	1	HT
2.	CSR27-192	1	HT
3.	CST7-1	1	HT
4.	CSR2K-242	1	HT
5.	PNL4-35-20-4-1-4	1	HT
6.	NDRK11-1	1	HT
7.	NDRK11-3	3	T
8.	CR2814-2-4-3-1-1-1	3	T
9.	CSR2K-262	1	HT
10.	CR 2218-64-1-327-4-1	5	MT
11.	NDRK 11-7	5	MT
12.	CSR2K-219	5	MT
13.	Kalanamak	5	MT
14.	NDRK 11-5	5	MT
15.	NDRK 11-6	5	MT
16.	NDRK 11-4	5	MT
17.	RAU 1-1648	5	MT
18.	IR 64	7	S

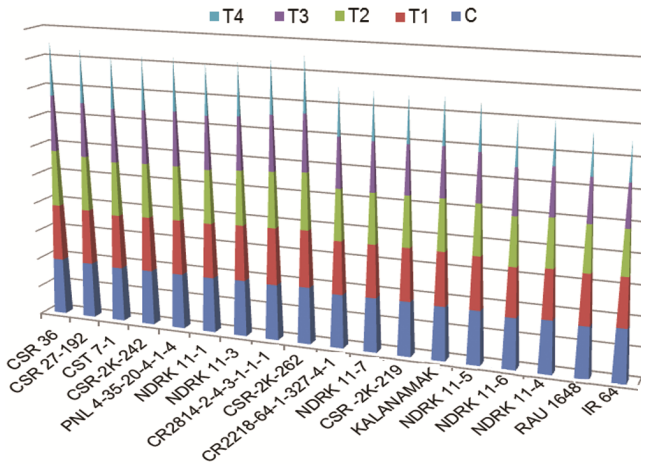

Fig. 1 — Effect of different level of salinity on germination percent of rice genotypes.

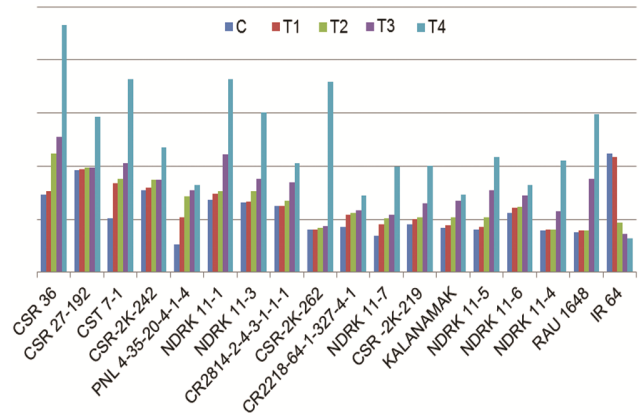

Fig. 2 — Effect of different salinity level on K/Na ratio at seedling stage of rice genotypes. (C: 0 ds/m, T1:2 ds/m, T2:4 ds/m, T3:6 ds/m and T4:8 ds/m).

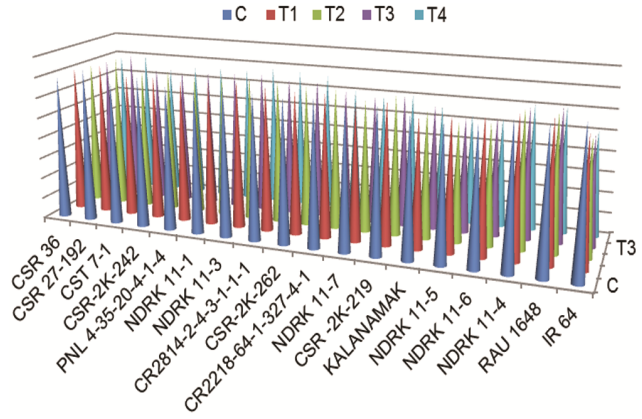

Fig. 3 — Effect of different level oof salinity on shoot length.

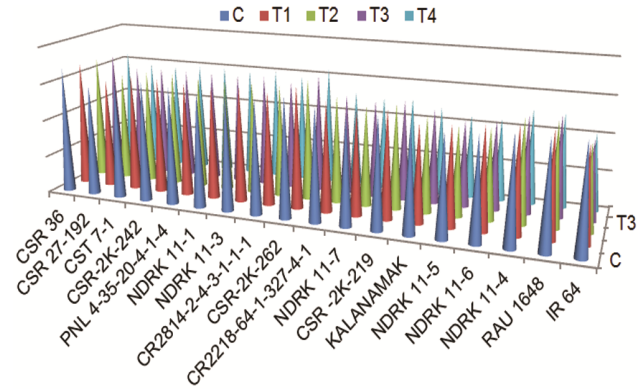

Fig. 4 — Effect of different level of salinity on root length.

Shoot fresh weight (SFW) and root fresh weight (RFW) also showed continuous decrease with increase in salt concentration. The percent reduction over control (%ROC) was recorded greater at 8 dSm^{-1} in all the genotypes. The highest decrease for SFW and RFW was recorded for IR64 showing its susceptible relation with salt. The genotypes CSR27-192, PNL4-35-20-4-1-4, NDRK11-1, NDRK11-3, CR2814-2-4-3-1-1-1, CSR2K-262, NDRK11-4 and RAU1-1648 showed better performance in terms of SFW and RFW than tolerant check CSR36 indicating their higher degree of tolerance to salinity (Fig. 5 & 6).

Shoot dry weight (SDW) and root dry weight (RDW) also followed a continuous reduction pattern with increasing salt concentration (Fig. 5 & 6). The reduction over control (%ROC) at 8 dSm^{-1} was recorded highest in all genotypes compared to lower treatment of salt stress. The highest reduction was observed in IR64 among all the genotypes under investigation. In case of SDW, the genotypes CST7-1, CSR2K-242 and NDRK11-4 performed better due to higher level of tolerance than tolerant check CSR36. In the case of RDW, only three genotypes performed inferiorly than tolerant check.

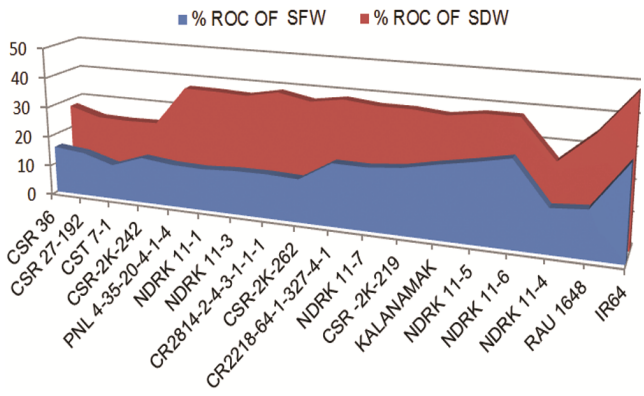

Fig. 5 — Representation of %ROC (reduction over control) of shoot fresh weight (SFW) and shoot dry weight (SDW) at EC 8 dSm^{-1} of rice genotypes.

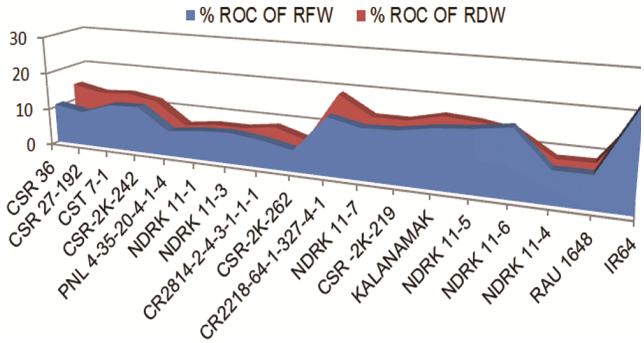

Fig. 6 — Representation of %ROC (reduction over control) of root fresh weight (RFW) and root dry weight (RDW) at EC 8 dSm^{-1} of rice genotypes.

Characterization and Screening of Rice Genotypes through SSR Marker for Salt Stress

SSR Polymorphisms and Similarity Coefficient

A panel of 44 SSR primer pairs used in the present study produced scorable, unambiguous markers. A total of 62 loci were amplified and found polymorphic. Average polymorphism percentage (PP) was 44.079. Altogether 410 amplified products representing allelic variants were generated by different primers with an average of 9.3 alleles per locus. Using the 44 primer pairs, a total of 185 shared and 225 unique allelic variants were generated in the present study (Table 5). Considering the number of alleles generated in conjunction with the level of polymorphism detected in the present study, the primers RM9, RM24, RM204, RM333, RM336, RM493 (Fig. 7), RM1287, RM3412, RM7025, RM8094 (Fig. 8), RM10793, RM25092, RM25217 and RM25519 appeared to be highly polymorphic and comparatively more informative primers.

The presence of unique alleles indicated that the materials used in this study are useful as a rich source of genetic diversity for effective utilization in rice breeding. The SSR product size ranged from 92 bps (RM10825) to 340 bp (RM10772). The level of polymorphism exhibited by each of the primer pairs was further assessed by calculating polymorphism information content (PIC), which reflects allele diversity and frequency of the markers among the entries (Table 5). From a perusal of the pertinent data, it is apparent that the PIC values were not uniform for all the primer pairs tested, revealing noticeable extent of variability in respect of simple sequence length polymorphism based allele diversity and frequency among the entries. Numerically, the value was found to vary from 0.392 in the case of primer RM 220 to 0.907 in the case of primer RM 336 with an average value of 0.767 across all the primers. The PIC value for co-dominant markers ranges from 0 to 1 and higher the PIC value, more the marker is informative[19-21]. So, all the markers used in the study are highly informative except RM2 (PIC < 0.5). The PIC values observed in the present study are comparable to previous reports[22-23].

Similarity index or similarity coefficients determines how closely the current plant community

Table 5 — Analysis of primer pairs used for the amplification of genomic DNA extracted from 18 rice entries

Primers	No. of locus	Size of alleles (bp)	Average size (bp)	Diff (bp)	No. of alleles	No. of unique alleles	No. of shared alleles	Polymorphism percentage	PIC	DC	NDC
RM2	2	140.00-165.63	153	26	5	2	3	40	0.57	0.64	0.359
RM4	1	150.00-175.00	163	25	9	4	5	44.44	0.86	0.83	0.17
RM5	1	108.33-118.75	114	10	6	1	5	16.67	0.79	0.837	0.163
RM9	3	125.00-250.00	188	125	14	7	7	50	0.8	0.98	0.02
RM11	1	131.58-158.82	145	27	8	4	4	50	0.81	0.856	0.144
RM14	2	176.32-200.00	188	24	7	3	4	42.86	0.75	0.797	0.203
RM20	2	217.86-287.50	253	70	15	6	9	40	0.64	0.954	0.046
RM24	2	162.50-204.17	183	42	10	5	5	50	0.84	0.902	0.098
RM25	1	142.86-175.00	159	32	9	3	6	33.33	0.86	0.915	0.085
RM113	1	148.00-155.00	152	7	4	1	3	25	0.64	0.68	0.32
RM140	1	250.00-290.91	270	41	6	3	3	50	0.74	0.784	0.216
RM204	1	108.70-171.43	140	63	10	4	6	40	0.88	0.928	0.072
RM210	2	115.79-176.67	146	61	17	9	8	52.94	0.67	0.98	0.02
RM219	1	206.67-240.00	223	33	8	4	4	50	0.81	0.856	0.144
RM220	3	116.00-292.31	204	176	16	6	10	37.5	0.39	0.974	0.026
RM223	2	138.10-165.79	152	28	7	3	4	42.86	0.75	0.817	0.183
RM242	1	200.00-238.46	219	38	8	3	5	37.5	0.83	0.869	0.131
RM253	2	132.50-171.88	152	39	10	6	4	60	0.73	0.863	0.137
RM261	1	128.00-146.00	137	18	8	4	4	50	0.82	0.869	0.131
RM281	2	143.75-203.85	174	60	11	2	9	18.18	0.66	0.935	0.065
RM292	1	158.33-169.44	164	11	4	2	2	50	0.59	0.627	0.372
RM333	1	162.50-216.67	190	54	12	7	5	58.33	0.9	0.954	0.046
RM336	1	142.86-188.24	166	45	12	6	6	50	0.91	0.961	0.039
RM490	1	102.00-122.00	112	20	9	4	5	44.44	0.85	0.85	0.15
RM493	2	197.37-244.44	221	47	15	8	7	53.33	0.77	0.98	0.02
RM562	1	200.00-247.22	224	47	9	5	4	55.56	0.85	0.889	0.111
RM1287	2	125.93-164.29	145	38	12	7	5	58.33	0.81	0.915	0.085
RM3395	1	101.79-141.07	121	39	9	5	4	55.56	0.84	0.889	0.111
RM3412	2	205.26-247.37	226	42	11	7	4	63.64	0.85	0.935	0.065
RM7025	1	132.61-168.42	151	36	10	5	5	50	0.9	0.948	0.052
RM8094	1	256.67-296.67	277	40	10	4	6	40	0.9	0.948	0.052
RM10665	1	262.50-295.83	279	33	5	1	4	20	0.7	0.732	0.268
RM10694	1	203.33-246.67	225	43	9	4	5	44.44	0.86	0.908	0.091
RM10745	1	171.05-184.21	178	13	5	3	2	60	0.63	0.667	0.333
RM10748	1	103.13-128.13	116	25	9	5	4	55.56	0.85	0.895	0.104
RM10764	1	211.54-326.32	269	115	7	2	5	28.57	0.82	0.863	0.137
RM10772	1	336.96-343.48	340	7	4	2	2	50	0.59	0.569	0.431
RM10793	2	132.35-208.33	170	76	13	5	8	38.46	0.66	0.961	0.039
RM10825	1	85.000-98.330	92	13	6	2	4	33.33	0.73	0.908	0.091
RM10864	1	150.00-168.42	159	18	4	1	3	25	0.67	0.712	0.287
RM11008	2	217.65-267.86	243	50	13	4	9	30.77	0.71	0.993	0.006
RM25092	2	137.50-189.47	163	52	14	6	8	42.86	0.82	0.948	0.052
RM25217	1	241.18-328.26	285	87	10	4	6	40	0.88	0.928	0.072
RM25519	1	190.00-239.29	215	49	10	6	4	60	0.85	0.902	0.098

resembles either the potential natural community or some other reference community[24-26]. The similarity index provides the distinct measurement in germplasm screening and diversity analysis.

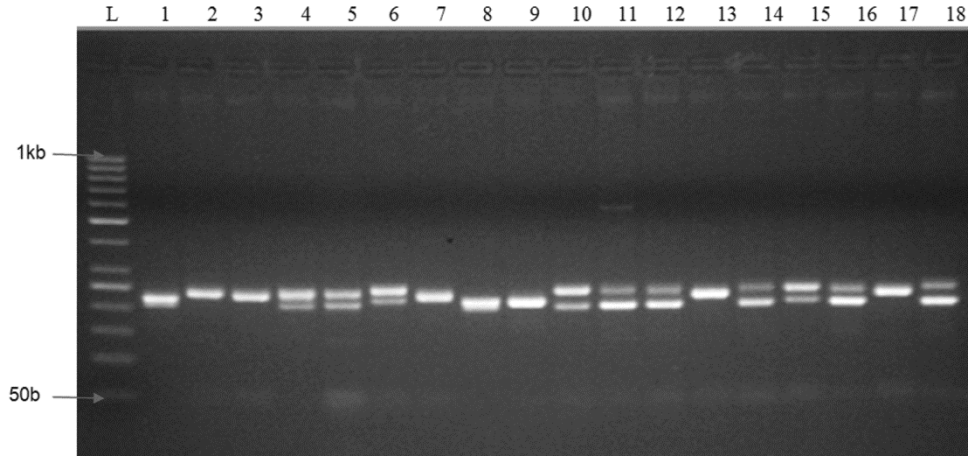

Fig. 7 — Amplification of region of genomic DNA extracted from rice grain by primer RM 493 used in the study.

1. CSR36	4. CSR2K-242	7. NDRK11-3	10. CR2218-64-1-327-4-1	13. KALANAMAK	16. NDRK11-4
2. CSR27-192	5. PNL4-35-20-4-1-4	8. CR2814-2-4-3-1-1-1	11. NDRK11-7	14. NDRK11-5	17. RAU1-1648
3. CST7-1	6. NDRK11-1	9. CSR2K-262	12. CSR2K-219	15. NDRK11-6	18. IR64

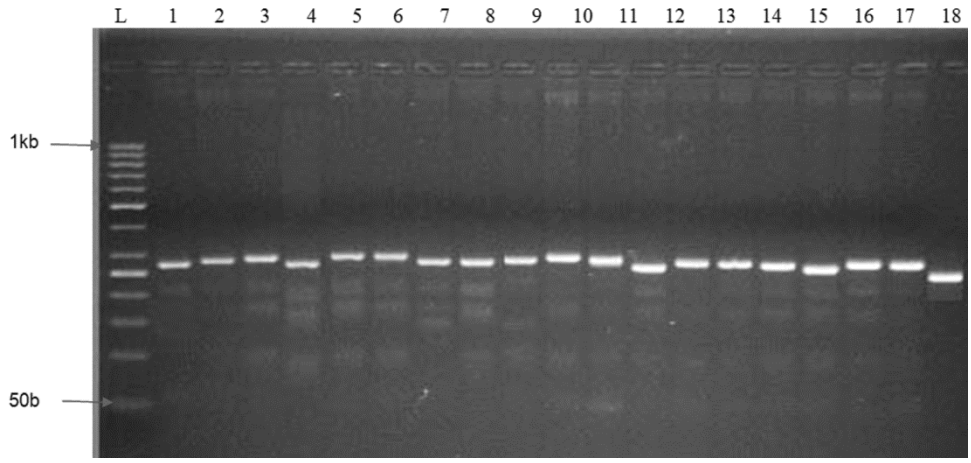

Fig. 8 — Amplification of region of genomic DNA extracted from rice grain by primer RM 8094 used in the study.

1. CSR36	4. CSR2K-242	7. NDRK11-3	10. CR2218-64-1-327-4-1	13. KALANAMAK	16. NDRK11-4
2. CSR27-192	5. PNL4-35-20-4-1-4	8. CR2814-2-4-3-1-1-1	11. NDRK11-7	14. NDRK11-5	17. RAU1-1648
3. CST7-1	6. NDRK11-1	9. CSR2K-262	12. CSR2K-219	15. NDRK11-6	18. IR64

Similarity index value was obtained for each pair wise comparisons among the 18 rice genotypes (Table 6). The similarity coefficients based on 44 SSR loci ranged from 0.75 to 0.85. Among the 18 rice genotypes, the highest similarity was observed between genotype CSR2K-242 and genotype PNL4-35-20-4-1-4. The lowest similarity index (0.75) was observed between IR64 and NDRK11-1, IR64 and CR2218-64-1-327-4-1 and IR64 and NDRK11-7. Similar inference has been derived in the studies conducted on the molecular markers including SSR marker based divergence analysis in rice by earlier researchers[27-29].

Cluster Analysis

The multivariate nature of SSR makers has the unambiguous advantage of discriminating genotypes more precisely[30-32]. The cluster analysis revealed allelic richness of three clusters (Fig. 9) for various sizes at a similarity coefficient level of 0.77. SSR analysis resulted in a more definitive separation of cluster of genotypes indicating a higher level of efficiency of SSR markers for the accurate determination of relationships between accessions that are too close[33]. Grouping based on SSR markers, in general, agreed with the parental pedigree information providing indispensable information regarding the

Table 6 — Estimates of 44 SSR primer pairs based SM similarity coefficients among 18 rice entries used in the present study.

	CSR 36	CSR 27-192	CST 7-1	CSR 2K-242	PNL 4-35-20	NDR K11-1	NDR K11-3	CR2814-2-4-3-1-	CSR 2K-262	CR2218-64-1-327	NDR K11-7	CSR2 K-219	KALA NAMAK	NDR K11-5	NDR K11-6	NDR K11-4	RAU 1-1648
CSR27-192	0.8																
CST7-1	0.81	0.82															
CSR2K-242	0.79	0.78	0.79														
PNL4-35-20-4	0.79	0.79	0.84	0.85													
NDRK11-1	0.79	0.79	0.8	0.83	0.84												
NDRK11-3	0.79	0.8	0.81	0.82	0.82	0.81											
CR2814-2-4-3-	0.78	0.78	0.79	0.8	0.8	0.79	0.84										
CSR2K-262	0.8	0.8	0.79	0.8	0.8	0.77	0.81	0.84									
CR2218-64-1-	0.77	0.79	0.78	0.78	0.78	0.78	0.78	0.79	0.8								
NDRK11-7	0.78	0.79	0.76	0.78	0.79	0.77	0.77	0.78	0.8	0.81							
CSR2K-219	0.78	0.77	0.78	0.79	0.78	0.79	0.79	0.8	0.8	0.81	0.84						
KALANAMAK	0.77	0.78	0.78	0.77	0.76	0.77	0.78	0.78	0.79	0.79	0.8	0.83					
NDRK11-5	0.77	0.77	0.77	0.76	0.76	0.76	0.77	0.78	0.77	0.77	0.77	0.8	0.81				
NDRK11-6	0.77	0.78	0.77	0.77	0.76	0.78	0.76	0.77	0.77	0.77	0.77	0.8	0.81	0.82			
NDRK11-4	0.78	0.77	0.77	0.78	0.78	0.78	0.78	0.78	0.79	0.8	0.78	0.84	0.8	0.8	0.8		
RAU1-1648	0.79	0.79	0.77	0.8	0.79	0.79	0.78	0.79	0.78	0.78	0.78	0.79	0.79	0.79	0.79	0.8	
IR64	0.78	0.8	0.79	0.77	0.78	0.75	0.78	0.78	0.78	0.75	0.75	0.77	0.76	0.8	0.78	0.78	0.79

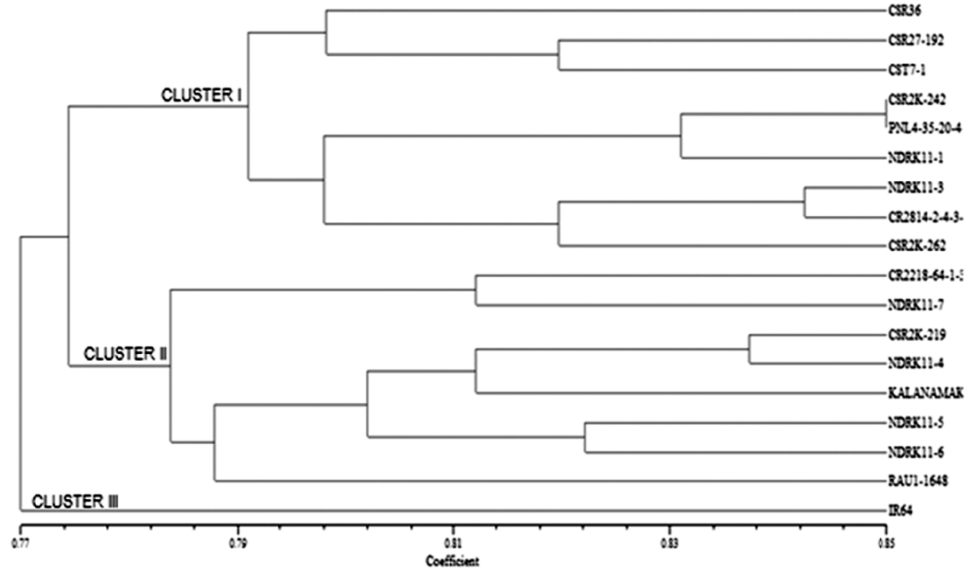

Fig. 9 — An UPGMA dendogram showing the genetic relationship among 18 rice genotypes based on 44 SSR markers profile.

genetic diversity among the genotypes[34]. Varieties and lines sharing the common ancestry were clustered in to the same group, indicating the efficiency of SSR markers in detecting the genetic diversity in rice[35]. To estimate the genetic relatedness among the 18 rice genotypes, similarity analysis was done and dendogram showing the genetic relatedness among 18 rice genotypes was constructed (Fig. 10). Cluster I was the largest with 9 genotypes (CSR36, CSR27-192, CST7-1, CSR2K-242, PNL4-35-20-4-1-4, NDRK11-1, NDRK11-3, CR2814-2-4-3-1-1-1, CSR2K-262) with all highly tolerant and tolerant genotypes identified by visual scoring at seedling stage, Cluster II comprised of 8 genotypes (CR2218-64-1-327-4-1, NDRK11-7, CSR2K-219, Kalanamak, NDRK11-5, NDRK11-6, NDRK11-4, RAU1-1648) with all tolerant and moderately tolerant at seedling stage and Cluster III comprised of 1 genotype (IR64) identified as susceptible one.

The multivariate nature of SSR makers has the unambiguous advantage of discriminating genotypes more precisely. The cluster analysis revealed allelic richness of three clusters (Fig. 9) for various sizes at a similarity coefficient level of 0.77. SSR analysis resulted in a more definitive separation of cluster of genotypes indicating a higher level of efficiency of SSR markers for the accurate determination of relationships between accessions that are too close. Grouping based on SSR markers, in general, agreed with the parental pedigree information providing indispensable information regarding the genetic diversity among the genotypes. Varieties and lines sharing the common ancestry were clustered in to the same group, indicating the efficiency of SSR markers in detecting the genetic diversity in rice. To estimate the genetic relatedness among the 18 rice genotypes, similarity analysis was done

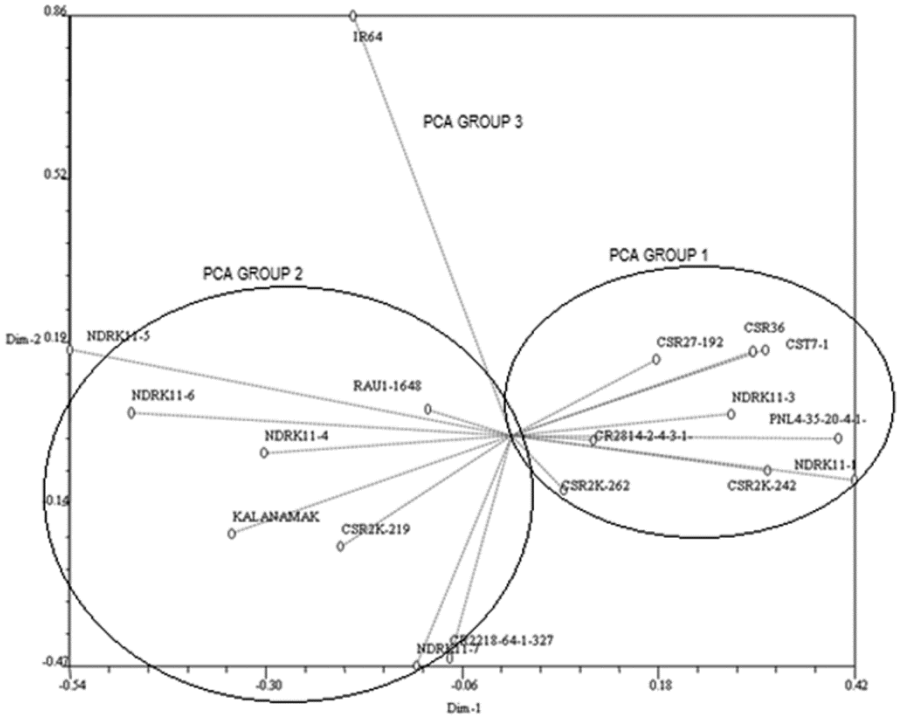

Fig. 10 — 2-D plot of principle component analysis based on polymorphisms of 44 SSR markers of rice genotypes.

and dendogram showing the genetic relatedness among 18 rice genotypes was constructed (Fig. 10). Cluster I was the largest with 9 genotypes (CSR36, CSR27-192, CST7-1, CSR2K-242, PNL4-35-20-4-1-4, NDRK11-1, NDRK11-3, CR2814-2-4-3-1-1-1, CSR2K-262) with all highly tolerant and tolerant genotypes identified by visual scoring at seedling stage, Cluster II comprised of 8 genotypes (CR2218-64-1-327-4-1, NDRK11-7, CSR2K-219, Kalanamak, NDRK11-5, NDRK11-6, NDRK11-4, RAU1-1648) with all tolerant and moderately tolerant at seedling stage and Cluster III comprised of 1 genotype (IR64) identified as susceptible one.

Principle component analysis (PCA) was applied to the raw data obtained from SSR '1' and '0' matrix which partitioned the sample into three distinct groups. PCA group 1 includes 9 samples, PCA group 2 includes 8 samples and PCA group 3 includes only one sample.

Discussion

The present study showed remarkable differences in salt tolerance among genotypes for germination percent, root length and shoot length, root fresh weight and root dry weight, shoot fresh weight and shoot dry weight, and K/Na ratio. The osmotic effect due to salinity was the main inhibitory factor that reduced germination. It was also reported that the reduction in seed germination and seedling vigour under salinity stress to the adverse influence on the activity of key enzymes like amylase, RNase and protease in the endosperm and consequent depletion of food reserves in embryo[36-38]. Germination percentage was lower in salinized condition in all the genotypes compared to plants grown in non-salinized condition. In the present study, germination percentage followed a continuous decrease with increasing salinity treatment. The highest decrease in germination percent at 8 dSm^{-1} salinity level was observed in IR64, while lowest decrease in germination percentage was recorded for CSR2K-262 (Fig. 1). Abeysiriwardena and De (2004) also reported reduction in germination per cent with increasing salinity and observed that tolerant cultivars recorded higher germination per cent than the sensitive cultivars. Relatively higher amount of K than Na is probably required in the leaves for the protection of growing plants from the toxic effect of Na ion[39]. In the present investigation a continuous increase in K/Na ratio was observed with increasing salt concentration in all the genotypes except IR64 in which a decrease in K/Na ration was found with increase in salinity which confirms its susceptibility to salt stress. In case of shoot length, tolerance

check CSR36 recorded least reduction, while the genotypes CSR2K-262, PNL4-35-20-4-1-4, CSR27-192, NDRK11-1, NDRK11-3, CR2814-2-4-3-1-1-1 and CSR2K-262 showed higher level of tolerance than tolerant check in terms of root length at seedling stage (Fig. 2). With increase of salinity, reduction of root length, shoot length, dry weight of root and dry weight of shoot was also reported earlier (Roy *et al*, 2002). Shoot dry weight (SDW) and root dry weight (RDW) also followed a continuous reduction pattern with increasing salt concentration. Awada *et al* (2010) observed that relative root biomass in rice genotypes was significantly lower at higher salt concentration.

The SSR primer based analysis that revealed the polymorphism on the basis of variation in the length of simple sequence repeats was an efficient tool for differentiation of entries and diversity analysis[40-41]. Using 44 SSR primer pairs for molecular characterization of a set of 18 entries, amplification was successfully achieved with all the primer pairs. Polymorphism was recognized on the basis of presence or absence of bands, besides variation in number and position of bands. In general, marker detecting greater number of alleles per locus detected more number of unique alleles in accordance with the earlier reports.

Using 44 SSR primer pairs, a total of 410 allelic variants were detected at 62 loci with an average of 9.3 alleles per locus among 18 entries characterized under present investigation. Analysis of divergence pattern based on amplification pattern obtained with 44 SSR primer pairs allowed extent of genetic relatedness of tolerant and susceptible rice genotypes. Considering the number of alleles generated in conjunction with the level of polymorphism detected in the present study, the primers RM9, RM24, RM204, RM333, RM336, RM493, RM1287, RM3412, RM7025, RM8094, RM10793, RM25092, RM25217 and RM25519 appeared to be highly polymorphic, capable of distinguishing salt tolerant genotypes and comparatively more informative. The 2-D plot array of PCA is also appeared highly congruent with UPGMA cluster diagram.

Molecular and physiological characters are ultimate expression of genetic constitution of a variety for a QTL trait. So, considering diversity pattern and combining physiological parameters at seedling stage and molecular assessment of all the inbred lines taken in this study which was under evaluation for STRASA project can be considered as tolerance and can be used in further breeding programmes.

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